Experimental studies on the infectivity of non-culturable forms of *Campylobacter* spp. in chicks and mice

A. W. VAN DE GIESSEN[1]*, C. J. HEUVELMAN[1], T. ABEE[2] AND W. C. HAZELEGER[2]

[1] *Microbiological Laboratory for Health Protection, National Institute of Public Health and Environmental Protection, P.O. Box No. 1, 3720 BA Bilthoven, The Netherlands*
[2] *Department of Food Science, Wageningen Agricultural University, Wageningen, The Netherlands*

SUMMARY

The significance of non-culturable forms of *Campylobacter* spp., especially with regard to the epidemiology of this organism in poultry flocks, was explored. Two different experiments were conducted to produce non-culturable *Campylobacter* spp. and test their ability to colonize the animal gut. In the first experiment a mixture of 28 different strains of *Campylobacter* spp. from various sources was inoculated in both sterilized surface water and potassium phosphate buffer and stored at 4 °C. After *Campylobacter* spp. were no longer detectable by culture in the microcosms, the mixtures of non-culturable cells were used to challenge both chicks and mice. Recovery of non-culturable *Campylobacter* spp. from the animals was not successful at 4 weeks after administration. In the second experiment the survival of six individual strains of *Campylobacter* spp. in sterilized surface water at 4 °C was studied and the resulting non-culturable cells were used to challenge chicks. None of the campylobacter strains could be recovered from the chicks at 2 weeks after administration. We conclude that occurrence of non-culturable forms of *Campylobacter* spp. capable of colonizing chicks is not a common phenomenon and that non-culturable forms of *Campylobacter* spp. are likely to be insignificant for importantly to the epidemiology of the organism in Dutch broiler flocks.

INTRODUCTION

Campylobacter jejuni/coli is recognized as a common cause of human diarrhoeal illness in many developed countries. Sporadic cases constituting the vast bulk of the campylobacter infections have predominantly been associated with consumption of poultry meat [1–3]. It is generally recognized that a reduction in human campylobacteriosis can best be pursued by preventing campylobacter infection in poultry flocks [4]. Several studies have been conducted to identify the transmission routes involved in the infection of poultry flocks with *Campylobacter* spp. Evidence is increasing against vertical transmission of *Campylo-*

bacter spp. from parent flocks to progeny, suggesting that poultry flocks become infected from the environment [5–8]. Several environmental factors including the poultry house environment, old litter, other farm animals, domestic pets, rodents, insects, wild birds, farm workers, equipment and transport vehicles have all been cited as potential sources of infection [4, 9, 10]. However, in several on-farm studies, in which epidemiological typing of campylobacter isolates was performed, the exact source of campylobacter infection in broilers could not be established [5, 8].

The animal digestive tract is the natural habitat of *Campylobacter* spp. Once excreted in the environment the organism usually does not multiply due to its

* Author for correspondence.

rather high minimal growth temperature of approximately 30 °C [11]. Moreover, *Campylobacter* spp. are very sensitive to drying and thus the organism does not easily survive in non-aquatic environments [12]. Under unfavourable conditions the organism transforms from a motile spiral form into a non-motile coccoid form and becomes non-culturable. For several pathogens, including *Vibrio*, *Salmonella* and *Shigella* spp., prolonged survival in aquatic environments in a non-culturable but still viable phase has been described [13]. Moreover, recovery of viable non-culturable bacteria in animal experiments has been reported for some pathogens including *Vibrio cholerae* [14] and enterotoxic *Escherichia coli* [15]. Rollins and Colwell [16] demonstrated that non-culturable *C. jejuni* obtained in a stream water microcosm at 4 °C retained viability and claimed that recovery of such cells by animal passage was successful. Evidently, the occurrence of non-culturable forms of *Campylobacter* spp. capable of colonizing the animal intestinal tract may have significant epidemiological implications. Pearson and colleagues [6] attributed colonization of broiler chickens with *C. jejuni* to a water supply in which non-culturable forms of this organism were observed by direct immunofluorescence microscopy. In order to test the infectivity of non-culturable *Campylobacter* spp. several experimental studies have been conducted yielding conflicting results. Both Medema and colleagues [17] and Fearnley and colleagues [18] reported a failure of viable non-culturable *C. jejuni* to colonize chicks. Also, Beumer and colleagues [19] reported that non-culturable *C. jejuni* could not be recovered from either mice, rabbits or human volunteers up to 30 days after administration. Conversely, Jones and colleagues [20] described colonization of mice by non-culturable *C. jejuni* induced in pond water at 4 °C and Saha and colleagues [21] reported recovery of freeze-thaw-injured non-culturable *C. jejuni* from rats. More recently, Stern and colleagues [22] reported colonization of chicks by non-culturable *Campylobacter* spp. induced in phosphate buffered saline at 4 °C. The results of the latter study suggest that the occurrence of colonizing non-culturable forms of *Campylobacter* spp. may not be uncommon and that such forms may play a role in the transmission of the bacterium to poultry flocks.

The aim of the present study was to explore the significance of non-culturable forms of *Campylobacter* spp., especially with regard to the epidemiology of this organism in poultry flocks. In previous research by Hazeleger and colleagues [23], coccoid cells of *C. jejuni* formed at 4 °C showed characteristics, including intracellular/extracellular ATP-ratio and membrane fatty acid composition, comparable to those of spiral cells, whereas those of coccoid cells formed at 25 °C were clearly different. Therefore, as a follow-up, in the present study survival experiments were conducted at 4 °C and these included the strains as well as the aquatic environment used in the experiments of Hazeleger and colleagues [23]. Two different experiments were carried out. In order to link our study to those of Stern and colleagues [22] and Jones and colleagues [20], in the first experiment a mixture of 28 different strains of *Campylobacter* spp. was inoculated in both surface water and potassium phosphate buffer and the non-culturable cells obtained were tested for their ability to colonize both chicks and mice. In the second experiment the survival of six individual strains of *Campylobacter* spp. in surface water was studied and the resulting non-culturable cells were tested for their ability to colonize chicks.

METHODS

Survival experiment A

In experiment A two types of microcosm were used: surface water and 50 mM potassium phosphate buffer (PPB [pH 7]). Surface water collected from an open storage reservoir for dune infiltrated water from which *Campylobacter* spp. previously had been isolated was stored at -20 °C. For use in the experiment, appropriate amounts of surface water and PPB were consecutively filtered through a Whatman general purpose filter (no. 1) and a $0.2\ \mu$m filter (Sartorius cellulose nitrate), sterilized at 121 °C for 15 min and stored at 4 °C until use. For the experiment 28 strains of *Campylobacter* spp. of various serotypes were selected including poultry, human, monkey, bovine, sewage and surface water isolates. Four of these strains were also used in the experiments of Hazeleger and colleagues [23] and one of these in the experiments of Medema and colleagues [17] as well (Table 1). The strains were maintained in Brucella Broth (BBL 11088) plus 15% glycerol at -70 °C. They were grown on campylobacter blood-free agar (Oxoid CM 739 without antibiotics) at 37 °C for 48 h in a microaerobic atmosphere (7% O_2, 10% CO_2 and 83% N_2). From each plate one colony was transferred onto a similar agar plate by swabbing the surface of the agar. After incubation under similar conditions, the entire growth from each plate was suspended in

Table 1. Campylobacter *strains used in experiment A*

Strain identification	Species	Serotype*	Source	Strain identification	Species	Serotype	Source
LA7	C. jejuni	O2	poultry	19532	C. jejuni	O29	human
D178	C. jejuni	O4	poultry	400	C. jejuni	O44	human
SA22.2	C. jejuni	O5	poultry	304	C. jejuni	O50	human
SA11.2	C. jejuni	O7	poultry	85252	C. jejuni	O59	human
VB1.9	C. coli	O14	poultry	2576	C. jejuni	NT	human
LB6	C. jejuni	O24, 54	poultry	AapA	C. jejuni	O3	monkey
SE32	C. jejuni	O49	poultry	ATCC 33560†	C. jejuni	NT	bovine
A63	C. jejuni	O49	poultry	LU104†	C. jejuni	NT	sewage
B31	C. jejuni	O59	poultry	197DZH	C. jejuni	O2	surface water
C1	C. jejuni	O60	poultry	272WMZ	C. jejuni	O5	surface water
B258†‡	C. jejuni	O59	poultry	163DZH	C. jejuni	O19	surface water
LU1010†	C. jejuni	NT§	poultry	151WMZ	C. jejuni	O24, 54	surface water
3746	C. jejuni	O16	human	140GWA	C. jejuni	U‖	surface water
579	C. jejuni	O18	human	107GWA	C. jejuni	U	surface water

* Passive haemagglutination assay of Penner and Hennesey [29].
† Strains used in the experiments of Hazeleger and colleagues [23].
‡ Strain used in the experiments of Medema and colleagues [17].
§ NT, not typed.
‖ U, untypeable.

2 ml of saline and centrifuged at 15000 g for 2 min. The cells were washed in 2 ml of the sterilized surface water, centrifuged and resuspended again in 2 ml of the same water. Then, the 28 suspensions of the different strains were mixed and 30 ml of the mixture was added to both 3 l of sterilized surface water (microcosm A1) and 3 l of PPB (microcosm B1). Finally, 1:10 dilutions of these microcosms were made by adding 30 ml of microcosm A1 and B1 to 270 ml of surface water (microcosm A2) and PPB (microcosm B2) respectively. The microcosms were held aerobically in sterilized Erlenmeyer flasks stationary at 4 °C in the dark. At the start of the incubation period and at 3–4 day intervals thereafter samples were taken from the microcosms and enumeration was carried out by spread plating on campylobacter blood-free agar. At low plate counts the microcosms were also tested by enrichment methodology. For this, samples of 1 ml and 5 ml of microcosm were added to 20 ml of campylobacter enrichment broth (Oxoid CM 67 with laked horse blood [Oxoid SR 48; 50 ml/l] and campylobacter growth supplement [Oxoid SR 84; 4 ml/l] without antibiotics), which was microaerobically incubated at 37 °C for 48 h. After incubation the broth was streaked on campylobacter blood-free agar, which was incubated as described above. When *Campylobacter* spp. were no longer detectable by either of these methods an additional portion of 50 ml of microcosm was filtered through a

0·2 µm filter (Sartorius) and the filter was transferred into 20 ml of campylobacter enrichment broth. When *Campylobacter* spp. were not detectable in 50 ml of the microcosm, animals were challenged within 3–4 weeks by portions of the microcosm as described below. Directly after challenging the animals another 150 ml of the microcosm was examined by filtration and enrichment as described for the 50 ml portions. At various stages during the survival experiment the morphology of the organisms was studied using phase-contrast microscopy.

Animal experiment A

Animal experiment A involved both chicks and mice. Fertile eggs were purchased from SPAFAS Inc., USA. The eggs were placed in an incubator which was then gassed with formalin and incubated under standard conditions. At day 19, the eggs were transferred aseptically to sterilized isolators ventilated with positive pressure filtered air. Different groups of eggs were placed in separate isolators. At the same day that the eggs hatched, a litter of newborn mice (BALB/c) and their mother, kept in sterilized plastic cages with filter tops, was placed in each of the isolators. Directly after hatching the chicks were provided with feed and water *ad libitum*. The animals were maintained at 32 °C throughout the experiments. Within 24 h after hatching the chicks were challenged orally with 100 µl

of either microcosm (A1, A2, B1 or B2) or saline by using a 1 ml tuberculin syringe. At the same time the infant mice were challenged with 50 μl of microcosm or saline by direct injection into the stomach. After 28 days the animals were killed. The caecal and intestinal contents of the chicks and mice respectively were directly streaked on both campylobacter blood-free agar and campylobacter blood-free selective agar (Oxoid CM 739 with cefoperazone [32 mg/l] and cycloheximide [100 mg/l]) which were micro-aerobically incubated for 48 h at 37 °C and 42 °C respectively. Additionally, portions of 1 g of caecum and intestine were transferred into campylobacter selective enrichment broth (as described above with Preston selective supplement [Oxoid SR 117; 4 ml/l]) which was microaerobically incubated at 42 °C for 48 h. After that, the broth was streaked on campylobacter blood-free selective agar which was incubated under the same conditions. Characteristic colonies were examined under a phase-contrast microscope for typical campylobacter morphology and motility.

Survival experiment B

Six strains of *Campylobacter* spp. were used including two strains of poultry origin (5C-O and 814-9-O) of which non-culturable cells had been found to colonize low numbers of 1-week-old chicks in experiments of Stern and colleagues [22]. Also, two strains of human and poultry origin respectively (85Y354 and B258) were included of which low numbers (10 c.f.u. and 26 c.f.u. respectively) of freshly cultured cells had been found to colonize 1-week-old chicks in the same animal model (van de Giessen and colleagues, un-published observations). Strain B258 was also used in the experiments of Hazeleger and colleagues [23]. Moreover, this strain was used in the experiments of Medema and colleagues [17] showing maintenance of viability (as indicated by substrate responsiveness in direct viable counts) after loss of culturability when kept in surface water at 25 °C. Finally, two strains (LU104 and 197DZH) isolated from sewage and surface water respectively were included. Strain LU104 was also used in the experiments of Hazeleger and colleagues [23]. The strains were grown on campylobacter blood-free agar. Then, from each plate one colony was transferred into 10 ml of Brain Heart Infusion (BHI) broth which was incubated in a microaerobic atmosphere at 37 °C for 48 h. After incubation the cultures were centrifuged at 5000 g for 15 min and each pellet was resuspended in 5 ml of

sterilized surface water described under experiment A. Finally, each of these suspensions was added to 300 ml of sterilized surface water. The six microcosms obtained were held aerobically in sterilized Erlenmeyer flasks stationary at 4 °C in the dark. Plate counts, enrichment testing and microscopy were conducted as described for experiment A. When *Campylobacter* spp. were not detectable in 50 ml of a microcosm, chicks were challenged within 1 week by portions of the microcosm as described below. Additionally, on the day of challenging, an acridine orange direct count (AODC) of the cells in the microcosm was carried out as described by Hobbie and colleagues [24].

Animal experiment B

Newly hatched chicks were purchased from Intervet B.V., The Netherlands, and transferred into isolators in which they were kept under conditions as described for experiment A. The next day the chicks were challenged orally with 100 μl of either microcosm, saline or a 24 h culture of one of the *Campylobacter* spp. strains in BHI (positive controls). However, since four strains became non-culturable at the same time, one group of chicks was challenged by a mixture of non-culturable cells of these four strains. For this, 2 ml of each of the respective microcosms were mixed and the chicks were challenged with 200 μl of this mixture. After 14 days the chicks were killed and their caecal contents were examined for *Campylobacter* spp. as described for experiment A.

RESULTS
Experiment A

The results of experiment A are presented in Table 2. Initial plate counts in microcosms A1/B1 and A2/B2 were $10^{6 \cdot 3}$ and $10^{5 \cdot 3}$ respectively. At that time, however, a large variety of cell forms was observed in the cosms, ranging from motile spiral to non-motile coccoid forms, the majority of them being coccoid, and probably non-culturable forms. During the survival experiment the culturability of the mixture of *Campylobacter* spp. strains in microcosms A1, A2, B1 and B2 declined and the cells became non-culturable after 4, 3, 7 and 5 weeks respectively. At that stage, mainly coccoid forms and occasionally non-motile spiral and long filamentous forms were observed. When these mixtures of non-culturable *Campylobacter* spp. were used to challenge both chicks and mice, the organism could not be recovered at 4 weeks after administration.

Table 2. *Challenge of chicks and mice by a mixture of non-culturable* Campylobacter *spp. of 28 different strains*

Microcosm	Initial plate counts (c.f.u./ml)	Weeks at 4 °C before non-culturability	No. of chicks colonized/no. of chicks challenged	No. of mice colonized/no. of mice challenged
Surface water (A1)	$10^{6\cdot3}$	4	0/6	0/4
Surface water (A2)	$10^{5\cdot3}$	3	0/5	0/6
PBB* (B1)	$10^{6\cdot3}$	7	0/5	0/6
PBB (B2)	$10^{5\cdot3}$	5	0/5	0/7
Saline	—	—	0/5	0/4

* PBB, 50 mM potassium phosphate buffer (pH 7).

Table 3. *Challenge of chicks by non-culturable* Campylobacter *spp. of six separate strains*

Strain identification	Species	Serotype*	Source	Initial plate counts in cosm (c.f.u./ml)	Weeks at 4 °C before non-culturability	No. of chicks colonized/no. of chicks challenged		
						Non-culturable cells†	Freshly cultured cells‡	Saline
85Y354	*C. coli*	NT§	human	$10^{7\cdot0}$	4	0/7	7/7	0/5
B258‖ᶜ	*C. jejuni*	NT	poultry	$10^{6\cdot6}$	6	0/7	6/7	0/6
5C-O**	*C. jejuni*	O23, 36	poultry	$10^{6\cdot8}$	7	0/5	5/5	0/5
814-9-O**	*C. jejuni*	O4, 16	poultry	$10^{6\cdot0}$	7	0/5	7/7	—
LU104‖	*C. jejuni*	NT	sewage	$10^{7\cdot1}$	7	0/5	5/5	—
197DZH	*C. jejuni*	O2	surface water	$10^{7\cdot4}$	7	0/5	6/6	—

* Passive haemagglutination assay of Penner and Hennesey [29].
† Inoculum per chick: $10^{5\cdot7}$–$10^{6\cdot2}$ cells (based on AODC).
‡ Inoculum per chick: 10^6–10^8 cells (based on plate counts).
§ NT: not typed.
‖ Strains used in the experiments of Hazeleger and colleagues [23].
ᶜ Strain used in the experiments of Medema and colleagues [17].
** Strains used in the experiments of Stern and colleagues [22].

Experiment B

The results of experiment B are presented in Table 3. Initial plate counts in the six different microcosms varied from $10^{6\cdot0}$–$10^{7\cdot4}$. Survival curves of separate strains showed a decrease in culturability resulting in non-culturable cells after 4–7 weeks. Microscopy revealed a gradual transition from motile spiral forms to coccoid forms during the survival experiment. Again, some non-motile spiral forms could be observed sporadically among the coccoid forms in the non-culturable suspensions. The inoculum of non-culturable cells varied from $10^{5\cdot7}$–$10^{6\cdot2}$ cells per chick (as determined by AODC). The inoculum of freshly cultured cells varied from 10^6–10^8 cells per chick (as determined by plate counts). At 2 weeks after administration *Campylobacter* spp. could not be recovered from any of the chicks challenged with non-culturable cells, whereas freshly cultured cells of all strains were found capable of colonizing chicks.

DISCUSSION

Several *in vitro* techniques have been described to assess the viability of non-culturable forms of *Campylobacter* spp. induced in aquatic environments [16–18]. However, the value of these techniques is questionable since up to now 'viability' of non-culturable cells, as indicated by one of these techniques, could not be confirmed by resuscitation of these cells under favourable conditions. Moreover, coccoid forms of *Campylobacter* spp. may retain various metabolic activities for a considerable time after loss of

culturability during degeneration [23]. Therefore, the ultimate test for establishing the viability of non-culturable forms of *Campylobacter* spp. is the ability of the cells to multiply in the intestinal tract of a host animal.

The present study involved different approaches for producing non-culturable *Campylobacter* spp. and testing their ability to colonize animals. The experimental conditions were chosen in such a way as to maximize the chance that viable non-culturable cells were formed and detected. In the first experiment a mixture of strains was inoculated in both surface water and PPB in order to test a large variety of strains in different aquatic environments. Since in the second experiment several individual strains were tested, only surface water was used as microcosm. In the first experiment, microcosms were inoculated with cells ranging from motile spiral to non-motile coccoid forms, due to cultivation on agar plates [25]. Therefore, it is likely that the inocula comprised cells at different levels of metabolic activity or degeneration. In the second experiment microcosms were inoculated with freshly cultured, morphologically uniform, spiral-shaped cells. During this survival experiment the expected gradual transition to a predominance of coccoid forms was observed [16, 26]. Both survival experiments were carried out at 4 °C, since up to now recovery of non-culturable *Campylobacter* spp. formed in aquatic environments has only been reported to be successful when the organisms were incubated at this temperature [16, 20, 22]. Moreover, previous research by Hazeleger and colleagues [23] revealed that coccoid cells of *Campylobacter* spp. formed at 4 °C show characteristics comparable to those of spiral cells. Indeed, the results of ATP-measurements in microcosms A1 and B1 during survival experiment A (data not shown) are in agreement with previous results [23], indicating that the intracellular ATP levels of the cells remained generally constant while culturability decreased. Culturability of the *Campylobacter* spp. mixtures in PPB clearly exceeded culturability in surface water, which may be due to the presence of small amounts of salts (reducing osmotic shock) and the control of pH (pH 7) in the buffer. Culturability of the mixtures may not reflect culturability of the individual component strains, which is inherent in the approach chosen. However, the *Campylobacter* spp. mixtures in surface water became non-culturable after 3–4 weeks, and thus large differences in survival in surface water between the different strains are not likely to have

occurred. Finally, about 75 % of each microcosm (except for microcosms A1 and B1) was used to verify a lack of proliferating capacity directly after animal challenges. Recovery of non-culturable *Campylobacter* spp. from the chicks or the mice was not successful 2 or 4 weeks after challenging, although for two strains (85Y354 and B258) the dose administered (Table 3) was estimated to be 40 000 to 100 000 times the colonizing dose of freshly cultured cells (A. W. van de Giessen and colleagues, unpublished observations).

Recovery experiments with non-culturable *Campylobacter* spp. obtained in aquatic environments have yielded conflicting results. The results of this study are in agreement with those of Medema and colleagues [17] and Fearnley and colleagues [18] who reported a lack of colonization of chicks by viable non-culturable *C. jejuni* obtained at 25 °C and 4 °C respectively. However, our findings are at variance with some other reports. Jones and colleagues [20] reported that non-culturable coccoid forms of *C. jejuni* obtained in pond water at 4 °C were capable of infecting mice but that this property may differ between strains. In our study, mixtures of non-culturable *Campylobacter* spp. obtained in surface water and PPB microcosms were not capable of colonizing suckling mice. This discrepancy may be explained by strain differences. The experimental approach of our study may best be compared to that of Stern and colleagues [22], who reported colonization of 1-week-old chicks by non-culturable *Campylobacter* spp. obtained in phosphate buffered saline held at 4 °C. Remarkably, two *C. jejuni* strains of which non-culturable cells were recovered from chicks by Stern and colleagues [22] were also tested in our experiments and non-culturable cells of these strains could not colonize chicks in our model. However, in contrast to our study, Stern and colleagues [22] used campylobacter selective enrichment instead of non-selective enrichment to test the culturability of the suspensions. Since *Campylobacter* spp. can suffer sub-lethal injury in aquatic environments which sensitizes them to selective agents [27, 28], the use of selective enrichment may have negatively influenced the culturability of their suspensions and may have erroneously led to the assumption that only non-culturable cells were used in the colonization experiments.

The results of this study cast serious doubts on the existence of non-culturable forms of *Campylobacter* spp. capable of colonizing chicks or mice. At the least, the results indicate that the occurrence of such forms

is not a common phenomenon. Having come to this conclusion, the question is whether non-culturable forms of *Campylobacter* spp. do have any epidemiological significance. As far as the epidemiology of *Campylobacter* spp. in broiler flocks is concerned, one could suggest that non-culturable *Campylobacter* spp. may play a role if many thousands of birds in the same flock are exposed to such organisms, for example via the old litter of previous flocks or via drinking water. In that case, colonization of a single bird by non-culturable forms may trigger colonization in other chicks. However, in The Netherlands broiler houses are cleaned and disinfected between successive broiler cycles excluding the transmission route via old litter.

The findings of Pearson and colleagues [6] suggest that infection of flocks by non-culturable *Campylobacter* spp. via untreated drinking water may occur. However, in this study like that of Stern and colleagues [22], only selective media were used for the culture of *Campylobacter* spp. from water samples. Moreover, in The Netherlands poultry flocks are provided with tap water (mains) and contamination of drinking water is not likely to occur. We conclude that, at least in The Netherlands, non-culturable forms of *Campylobacter* spp. are unlikely to play a part in the epidemiology of the organism in broiler flocks.

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